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(21) International Application Number: PCT/EP98/05362 (22) International Filing Date: 24 August 1998 (24.08.98) (30) Priority Data: 97306462.9 22 August 1997 (22.08.97) EP (71) Applicant (for all designated States except US): GIST-BROCADES B.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): YKEMA, Adriaantje [NL/NL]; Rijnsburgerwegh 185, NL-2334 BR Leiden (NL). STREEKSTRA, Hugo [NL/NL]; Weteringstraat 28-I, Amsterdam (NL). LUITEN, Rudolf, Gijsbertus, Marie [NL/NL]; Akkerhoombloem 26, NL-2317 KR Leiden (NL). (74) Agent: VISSER-LUIRINK, G.; Gist-Brocades B.V., Patents & Trademarks Dept., P.O. Box 1, NL-2600 MA Delft (NL).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: STATIN PRODUCTION BY FERMENTATION (57) Abstract A one-step biosynthesis process of pravastatin is disclosed. Fungal host cells that can produce compactin are transformed with a polynucleotide that encodes a foreign hydroxylase enzyme which is capable of converting the compactin into pravastatin. This can be cheaper and more efficient than previous two-stage fermentation processes (firstly a fungus to produce compactin, and then bacteria to hydroxylate the compactin to pravastatin).		

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STATIN PRODUCTION BY FERMENTATION

The present invention relates to fungal cells that
5 comprise a foreign polynucleotide that includes a hydroxylase
enzyme. The invention also relates to the production, by
such cells, of hydroxy-containing compound(s), that may be
useful for reducing cholesterol levels, by culturing these
cells.

10 The "statins" are a family of compounds that are
usually inhibitors of 3-hydroxy-3-methylbutyryl coenzyme
A(HMG-CoA) reductase, the rate-limiting enzyme in cholesterol
biosynthesis. As HMG-CoA reductase inhibitors, the statins
are able to reduce plasma cholesterol levels in various
15 mammalian species, including man, and are therefore effective
in the treatment of hypercholesterolemia.

Of all the statins known today, only two are
producable by fermentation *de novo*. These are lovastatin
(also called mevinolin, monacolin-K or mevacor) and compactin
20 (also known as mevastatin, or ML-236B). All the other
statins are produced by chemically or enzymatically
derivatising lovastatin or compactin. One of these is
pravastatin (also referred to as CS-514 in the art), which
has found favour recently as it is a more potent HMG-CoA
25 reductase inhibitor than lovastatin or compactin¹.

In order to produce pravastatin a two-step process

is required². Both steps involve separate fermentations as illustrated in the scheme below.

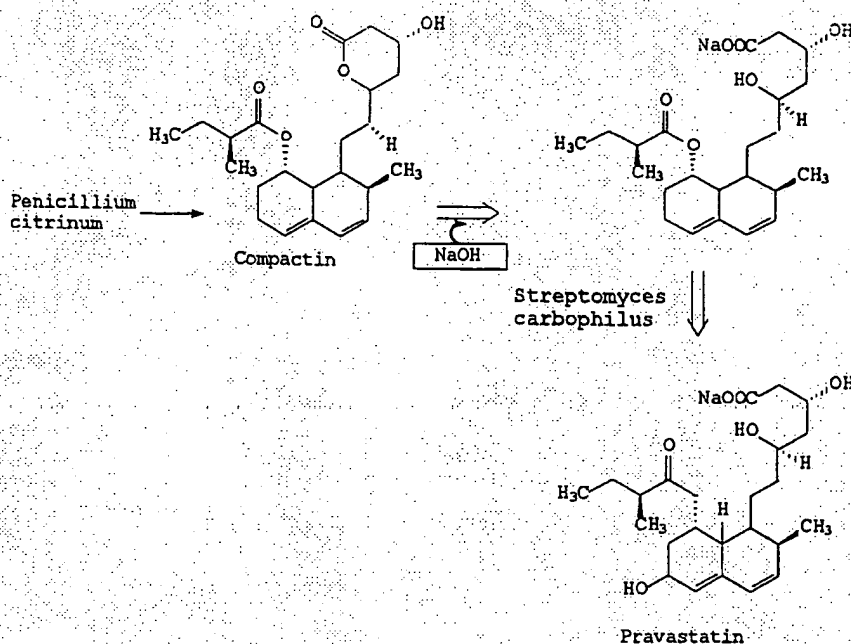


Figure 1. Pravastatin production by two fermentations.

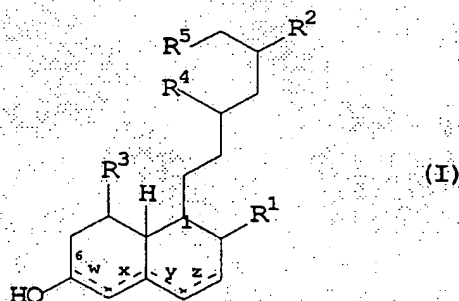
In the first step compactin is produced by a fungus, such as *P.citrinum*. After the lactone ring has been opened (by using sodium hydroxide) a second fermentation step takes place, converting the acid form of compactin into pravastatin by hydroxylation at the 6-position by a hydroxylase enzyme present in the bacterium *S. carbophilus*. This two-step fermentation process is required for a number of hydroxy-containing compounds that are related to statins or have a similar structure.

Clearly, one disadvantage with this process is that

two different fermentation methods need to be employed in order to make pravastatin (or other similar hydroxy-containing statin-like compounds). This reduces yields, and increases costs. One factor that has, until now, hindered progress towards a more efficient biosynthesis is the fact that compactin has mild anti-fungal properties which means that in the second hydroxylating stage manufacturers of pravastatin have used bacteria, such as of the genus *Streptomyces*. Indeed, many fungi have a low tolerance of compactin and therefore, today, the conversion of compactin to pravastatin is preferably effected using bacteria³.

The present invention seeks to solve, or at least mitigate, some of the problems encountered in prior art processes, and seeks to provide a more efficient process for preparing various hydroxy-containing statins, in particular pravastatin. Certainly, it would be desirable to be able to produce a hydroxy-containing statin by a more efficient method.

Thus according to a first aspect of the present invention, there is provided a process for the biosynthesis of compound of the general formula:



wherein:

each of R^1 and R^2 independently represent a hydrogen atom, a hydroxy, C_{1-10} alkyl, C_{1-10} alkoxy, C_{3-10} cycloalkyl or C_{6-10} aryl group or a C_{7-11} aralkyl group optionally containing

5 one or more hetero atoms;

R^3 represents R^1CO- or $R^1C(O)O-$;

each of R^4 and R^5 independently represent a hydrogen atom, $-COOR^1$ (except that then R^1 is not a hydroxy or alkoxy group), $-OR^1$ or $-COR^1$ or, when combined complete a
10 six-membered carbon ring having one or two oxygen heteroatoms;

wherein each of the alkyl, alkoxy, cycloalkyl, aryl and/or aralkyl groups can be optionally substituted with one or more halogen atoms, trifluoromethyl, hydroxy or C_{1-4} alkoxy
15 groups;

and w, x, y and z represent none, 1 or when taken together up to 2 double bonds;

or a salt and/or isomer thereof;

the process comprising culturing a fungal host cell
20 that expresses a foreign hydroxylase enzyme under conditions that allow biosynthesis to take place.

The alkyl and alkoxy groups (either alone or as part of a larger group) can be either straight or branched. Alkyl groups are preferably C_{1-4} alkyl groups, for example methyl or
25 ethyl. Alkoxy groups are preferably methoxy or ethoxy groups.

Preferred cycloalkyl groups are pentyl or hexyl groups. Suitable aryl groups include phenyl or naphthyl.

Preferably R^1 represents a hydrogen atom or a methyl

group. Preferably R^2 represents a hydroxy group. R^3 preferably represents $-OC(O)R^1$, where R^1 is a C_{1-10} alkyl group, for example a C_{1-4} alkyl group. In preferred compounds R^3 represents a 2-methyl butyrate group

5 $(-OC(O)CH(CH_3)C_2H_5)$.

R^4 preferably represents a hydroxy group and/or R^5 preferably represents $-COOH$. When R^4 and R^5 are not combined the compound of formula (I) is in the acid form: this can be converted to the lactone form by ring closure, so that R^4 and
10 R^5 when combined together can form a 6-membered ring (of carbon atoms) containing one or two oxygen heteroatoms. Here R^4 and R^5 combined preferably represent $-C(O)O-$.

It is preferred that two double bonds are present: in this situation, generally double bonds will be in the
15 locations w and y or x and z.

Compounds of general formula (II) in this specification are compounds of formula (I) except where the hydroxy (HO) group at the 6-position is replaced by a hydrogen atom (H). It will therefore be apparent that
20 compounds of formula (II) are precursors to those of formula (I), the conversion (from II to I) being effected by hydroxylation performed by the hydroxylase enzyme. Compounds of formula (II) therefore include compactin, which on hydroxylation form compounds of formula (I), which in the
25 case of compactin will give pravastatin.

For assistance, one of the preferred compounds of formula (II) is detailed in the following Table, illustrating the appropriate substituents.

TABLE 1

II	R ¹	R ³	R ⁴ and R ⁵	R ²	Double Bonds
Pravastatin (acid form)	CH ₃	OC(O)CH(CH ₃)C ₂ H ₅	OH and COOH	OH	x and z
Pravastatin (lactone)	CH ₃	OC(O)CH(CH ₃)C ₂ H ₅	-OC(O)-	OH	x and z

Compactin, of formula (I), is the same as pravastatin as shown in Table 1 above but does not possess the hydroxy (HO-) group at the 6-position.

The various isomers of compounds of formula (I) are included within the invention. This of course includes stereoisomers. Compounds of formula (I), or (II), preferably have the stereoisomerism of pravastatin or compactin, as appropriate.

Salts include acidic and/or basic salts formed with inorganic and/or organic bases. Non-toxic, pharmaceutically acceptable salts are preferred. These include ammonium salts, as well as alkali metals (e.g. sodium, potassium and lithium) or alkaline earth metal salts (calcium and/or magnesium).

Preferred compounds of formula (I) are HMG-CoA reductase inhibitors. Preferably they are hydroxylated derivatives of compounds of formula (II). Suitable compounds of formula (II) are polyketides. Polyketides are compounds that are biosynthesised internally by microorganisms, usually *de novo*, using low molecular weight precursors, for example acetyl moieties⁵. The acetyl moieties in turn can be obtained by the fungus from carbon sources such as starch, glucose, glycerol, etc.

It will be realised that the biosynthesis of compounds of formula (I) can be in the invention a one-step process, as opposed to a two-step process in the prior art. This is because the host cell employed is not only able to
5 produce compounds of formula (II), but it is also capable of converting, by hydroxylation, the compound of formula (II) to the compound of formula (I). Both these processes may take place inside the cell and the latter can be achieved by the foreign hydroxylase enzyme. In this manner, the process of
10 the invention can be significantly cheaper and more efficient than the 2-step processes used in the prior art.

The fungal host cell can preferably biosynthesise the compound of formula (I) *de novo*, that is to say from relatively small molecules: certainly, the fungal host cell
15 does not need to be fed or supplied with a compound of formula (II), in order to be able to biosynthesise compounds of formula (I), as it will usually be able to biosynthesise compounds of formula (II) itself.

The hydroxylase enzyme is "foreign" to the cell in
20 the sense that it is heterologous to that cell. In other words, the cell would not normally express that hydroxylase enzyme. Thus the naturally occurring or wild-type version of the fungal host cell will not express that hydroxylase enzyme (or at least not to a significant extent). Preferably, that
25 fungal host cell will not normally, such as in its native state, express such a hydroxylase enzyme at all, although preferably it will not express a hydroxylase enzyme that is capable of hydroxylating compounds of formula (II) to form compounds of formula (I). Nevertheless the fungal host cell

is preferably capable of biosynthesising a compound of formula (II), and by expression of the foreign hydroxylase enzyme can convert that compound into a compound of formula (I).

5 The fungal host cell can be any suitable fungus. However, preferably the fungus is of the genus *Paecilomyces*, *Eupenicillium*, *Trichoderma*, *Hypomyces*, *Penicillium*, *Monascus* or *Aspergillus*.

Preferred fungi are of the species *Penicillium*
10 *citrinum* or *Penicillium brevicompactum*. Other suitable strains that can produce compactin are additionally contemplated⁴. The most preferred fungus is *Penicillium citrinum* SANK 18767 that has been deposited under accession
nos. ATCC 38065 and NRRL-8082 or one of their descendants,
15 which may have been improved for compactin production (e.g. through classical strain improvement or recombinant techniques).

The fungal host cell can be cultured in any suitable culture medium, and under conditions known to a person
20 skilled in the art, that will allow the biosynthesis of compounds of formula (I). As the cell will usually be able to biosynthesise compounds of formula (II), such compounds are absent from this medium. Preferably, culture takes place under aerobic conditions, suitably in a culture medium that
25 provides the fungus with all the ingredients it needs to biosynthesise compounds of formula (I). A culture (e.g. liquid growth) medium that includes malt extract, glucose, peptone is usually sufficient. Agar can be used if the

medium is solid. As a guide a culture medium containing 2% malt extract, 2% glucose, 1% peptone (and 2% agar if solid) may be used.

The culture medium can contain any and all of the known nutrient materials for the fungus in order to allow biosynthesis to take place. For instance, an assimilable carbon source and nitrogen source are preferably present. For a carbon source, one can include glucose, glycerol, maltose, dextrin, dextrose, starch, lactose, sucrose, molasses, soybean oil, cotton seed oil etc. Glucose, dextrose and glycerol are preferred.

As a nitrogen source, one can mention soybean meal, peanut meal, cotton seed meal, fish meal, corn steep liquor, peptone, rice bran, meat extract, yeast, yeast extract, sodium nitrate, ammonium nitrate, ammonia and ammonium sulphate. Yeast extract is preferred. Certain inorganic salts may additionally be present, for example sodium chloride, phosphate ions, calcium carbonate, iron (II) sulphate as can vitamins and/or amino acids. A minor amount of other metal salts may be added, if necessary.

The carbon and/or nitrogen source can be supplied at the beginning of biosynthesis, for example as in a batch process, or alternatively (and preferably) the carbon and/or nitrogen source is administered continuously and/or continually, as described in International Patent Application No. PCT/EP98/01123 filed on 20 February 1998.

In carrying out fermentation, this may be conducted with aeration and/or agitation.

The temperature of the culture medium is preferably

from 20 to 30°C, such as from 24 to 26°C. The fungal host cell can be cultured from 20 to 240 hours, preferably from 48 to 216 hours.

5 The compound of formula (I) can then be extracted by using a solvent and/or chromatography. Preferably an organic solvent is used, for example ether, benzene, ethanol, ethyl acetate, chloroform, acetone or acetic acid. Preferably the solvent is a combination of ethanol and ethyl acetate, or methanol, and column chromatography is employed.

10 The hydroxylase enzyme can be a naturally occurring enzyme, or can be a synthetic enzyme, for example a mutant or variant of a natural enzyme. Mutants and variants can be prepared either by truncation, or by addition, deletion or substitution of one or more amino acids as is known in the art.

15 Preferred hydroxylase enzymes are from the species *Amycolata autotrophica* such as ATCC 35204, *Streptomyces californicus* such as ATCC 15436, *Amycolatopsis mediterranei* such as ATCC 21411, *Saccharothrix australensis* such as ATCC 31497, *Saccharopolyspora hirsuta* such as ATCC 27875, 27876 or 20501, *Saccharopolyspora erythraea* such as ATCC 11635, *Streptomyces carbophilus* such as FERM-BP1145 and *Streptomyces flavovirens* such as SANK 63684. However this can exclude a hydroxylase enzyme from *Streptomyces clavuligeris*.

25 The enzyme is suitably not capable of converting deacetoxycephalosporin C (DAOC) to diacetyl cephalosporin C (DAC).

The biosynthesis in the process of the first aspect can be accomplished by using host fungal cells which form the second aspect. The second aspect of the invention therefore relates to a fungal host cell comprising a polynucleotide
5 that encodes a foreign hydroxylase enzyme. The host cell is thus suitably able to express the foreign hydroxylase enzyme which can convert compounds of formula (II) (e.g. biosynthesised within the host cell) into compounds of formula (I). Preferably the host cell will be one that, in
10 the absence of the foreign hydroxylase gene, produces compactin.

A third aspect of the present invention relates to a vector comprising a polynucleotide comprising a sequence encoding a hydroxylase enzyme which is operably linked to a
15 fungal expression signal and/or fungal promoter. The vector is thus one that is effective in a fungal host cell. The polynucleotide can therefore be adapted so that, when inside a host fungal cell, it allows the enzyme to be expressed by that cell.

20 The promoter or expression signal(s) can be from any fungus as described for the first aspect. However, it does not necessarily have to be from the same genus or species as the host cell. Preferred promoters are from the genus *Aspergillus*, such as from the species *Aspergillus terreus* or
25 *Aspergillus nidulans*, or from *Penicillium*, such as from the species *P. chrysogenum* or *P. citrinum*.

The vector can be a plasmid or of bacteriophage origin. It may additionally comprise a terminator region:

this may also be from a fungus. Preferably the fungal expression signals (which include promoter and/or terminator) region are from a (e.g. highly or constitutively) expressed gene, such as a glycolytic gene, for example

- 5 3-phosphoglycerate kinase (*pgk*) or glucose-6-phosphate dehydrogenase (*gpd*). Preferably the terminator is from the same species as the promoter. The vector may further contain one or more additional sequences in order to optimise the production of compounds of formula (I). These sequences may
- 10 include, for example, a P450 reductase.

The vector will suitably also contain a selective or selectable marker or, if two or more vectors are used (such as in a co-transformation procedure) the marker can be in another vector. This marker may confer resistance to one or

15 more inhibitory substances (e.g. antibiotics) or the ability to utilise certain nutrients. For example, it may confer resistance to hygromycin or phleomycin. Alternatively a marker-free system can be employed¹⁴.

The vector can therefore be used to transform or

20 transfect a fungal cell in order to produce the cell of the second aspect. Transformation and/or transfection can be accomplished using known techniques. A fourth aspect of the invention therefore relates to a process for transfecting or transforming a host cell (such as to produce a cell of the

25 second aspect), the process comprising transforming or transfecting a fungal cell with a vector of the third aspect.

A fifth aspect of the present invention relates to polynucleotide comprising a sequence encoding a hydroxylase enzyme operably linked to a fungal expression signal and/or

fungal promoter.

A sixth aspect relates to a compound of formula (I) when produced by the process of the first aspect or a host cell of the second aspect. A seventh aspect of the invention
5 relates to a pharmaceutical composition comprising the compound of the sixth aspect and a pharmaceutically acceptable carrier or excipient.

Preferred features and characteristics of one aspect of the invention are applicable to another aspect *mutatis*
10 *mutandis*.

The invention will now be described by way of example only, with reference to the following Examples, which are provided for illustration and are not to be construed as being limiting on the invention.

EXAMPLESEXAMPLE 1Isolation of *Streptomyces carbophilus* hydroxylase gene.

Strain *S. carbophilus* deposited at the Fermentation
5 Research Institute, Ibaraki-ken, Japan under Accession No.
FERM-BP1145^{6,7} was grown at 28°C for 72 hrs in a liquid medium
containing:

	yeast extract	1g/l
	casamino acids	4g/l
10	glycerol	4g/l
	MgSO ₄	1g/l
	CaCl ₂	0.1g/l
	trace elements solution	
	(0.1% ZnSO ₄ ·7H ₂ O, 0.1% FeSO ₄ ·7H ₂ O,	
15	0.1% MnCl ₂ ·4H ₂ O)	2ml/l
	glycine (to a final concentration of 0.5%).	

The mycelium was then harvested by centrifugation,
washed once with 0.7% NaCl solution, and subjected to a total
DNA isolation procedure⁸.

20 For isolation of the compactin hydroxylase gene
the following 4 oligonucleotide primers were prepared based
on the published sequence⁹ (with minor adaptations to
facilitate cloning):

primer 8804 5'-CACCATGGCCGAGATGACAGAGAAAGCC-3'

25 primer 8805 5'-CAGGATCCCGCTCGGTCACCAGGTGACC-3'

Oligonucleotide combination 8804/8805 was used to amplify the hydroxylase gene from the *S. carbophilus* chromosomal DNA using PCR with the following characteristics: first cycle 3 minutes 96 °C, 30 seconds 60°C, 90 seconds 72°C; next 29 cycles of 20
5 seconds 96 °C, 30 seconds 60°C, 90 seconds 72°C; and finally one cycle of 7 minutes 72°C followed by quick cooling to 4°C.

A DNA fragment about 1.3 kb long was obtained containing the hydroxylase gene. The 1.3 kb blunt-end PCR fragment was inserted into the general cloning vector pCR-blunt
10 blunt (Stratagene, La Jolla, USA) resulting in plasmid pCRP450a.

EXAMPLE 2

Vector construction and transformation of *E. coli*

For the introduction of the hydroxylase gene into
15 different filamentous fungi a set of transformation vectors was constructed. These vectors were based on several general fungal expression vectors of the pAN series¹⁰, particularly pAN7-1 and pAN8-1.

pAN8-1 was digested with NcoI and SmaI and into it
20 ligated the 1.3 kb fragment encoding the hydroxylase gene, obtained by digestion of pCRP450a by EcoRV and partially by NcoI. The ligation mixture was introduced in *E. coli* strain XL1-Blue (electrocompetent cells, Stratagene, La Jolla, USA) using the technique recommended by the supplier. Thus plasmid
25 pANP450a was created and isolated, derived from insertion of the PCR fragment obtained from primer combination 8804/8805.

The identity of the plasmid constructs of *E. coli* transformants was confirmed by restriction enzyme analysis and DNA sequencing.

EXAMPLE 3

5 Transformation of *Penicillium citrinum*

Protoplast formation, transformation with plasmid DNA, and regeneration of *P. citrinum* was carried out similar to known methods for transforming filamentous fungi.¹¹

For fresh conidia formation *P. citrinum* ATCC 38065
10 was incubated on plates containing PDA (potato dextrose agar, Difco) at 26°C for 10 days. To 20 ml of YGG medium (6.6 g/l Yeast Nitrogen Base (Difco), 1.5 g/l citric acid, 6 g/l K₂HPO₄, 8 g/l KCl, 16 g/l glucose, and 2 g/l yeast extract) conidiospores were added to a final concentration of 10⁷
15 spores/ml. After incubation for 24 hrs at 26°C on a rotary shaker (280 rpm) 5 ml of germinated spores were inoculated in 100 ml of fresh YGG medium and incubated for another 16 hrs. Mycelium was collected by filtration through sterile Myracloth™ rinsed with and resuspended in 0.27 M CaCl₂, 0.6 M
20 NaCl at a concentration of approximately 1 g mycelium/20 ml. Protoplasts were prepared by incubation with NovoZym™ 234 at a final concentration of 5 mg/ml at 25°C with slow agitation (50-100 rpm) until maximum protoplastation. Protoplasts were separated from the mycelium by filtration through sterile
25 Myracloth™. The protoplast suspension was diluted (1:1) in STC buffer consisting of 1.2 M sorbitol, 10 mM Tris-HCl (pH

7.5), and 50 mM CaCl₂, and incubated on ice for 5-10 min. Protoplasts were collected by centrifugation (3000 rpm, 10 min, 0°C) and washed twice with STC. Finally, the protoplasts were resuspended at a final concentration of 10⁷-
5 10⁸/ml in STC.

Transformation of *P. citrinum* was carried out by cotransformation of plasmids pANP450a and pAN7-1 as follows: 40 µl of a solution containing 40 µg of plasmid DNA 20 µg of each plasmid) in the presence of 200 mM aurin tricarboxylic
10 acid was carefully mixed with 100 µl of 20% PEG 4000 in STC, followed by 200 µl protoplast-suspension and incubated for 20 min at 0°C. Next, 100 µl of 60% PEG 4000 in STC was added, followed by a 20 minute incubation at room temperature.

To obtain colonies of transformed fungi polyethylene
15 glycol-treated protoplast suspensions were diluted by addition of 5 ml of STC and the protoplasts were collected by centrifugation (10 min, 3000 rpm, 0°C) and resuspended in 500 µl of STC. About 100-200 µl of this suspension was plated onto osmotically stabilized (1.2 sorbitol) agar medium
20 to which Hygromycin B was added to a final concentration of 100 µg/ml.

Strains growing under selective conditions were left to sporulate (14 days), and purified by restreaking on PDA containing 100 µg/ml Hygromycin B. The presence of the
25 compactin hydroxylase gene sequence in the respective transformants was confirmed using the PCR technique as described in Example 1.

Strains proven to have obtained and integrated the transformed plasmid, including the complete hydroxylase gene were selected and named PRA201 through PRA 208.

EXAMPLE 4

5 Fermentative production of pravastatin by *Penicillium citrinum* transformant strains.

A batch of culture medium with the following composition was prepared:

	Glucose	75.0g
10	Yeast extract (Difco)	5.0g

The transformant strain numbers PRA201 to PRA208 were maintained on agar slants, and allowed to sporulate. The spores were collected from the agar medium, suspended in sterile water, and used to inoculate 100ml shake flasks,
15 containing 25ml of the culture medium.

Each strain was cultivated in duplicate shake flasks.

The production cultures were incubated at 25°C and 250rpm on an orbital shaker. After 5 days of incubation, the
20 cultures were sampled for statin production.

One ml of culture broth was transferred to a plastic tube, and diluted with 1ml methanol. The tube was stoppered, and subsequently shaken for 30 minutes on a Vortex shaker. Subsequently the tube was centrifuged to remove the
25 precipitate.

The supernatant fraction was analysed using HPLC.

The production of pravastatin was observed by the appearance of a peak at the characteristic retention time. The identity of this peak was confirmed by the addition of a pravastatin standard to the sample, which caused an increase of this
5 peak, and by analysis of the UV spectrum, which was identical to that of a genuine pravastatin standard.

The production levels (in mg/l) of the individual transformants is as follows:

	<u>Transformant</u>	<u>Production level</u> (in duplicate)
10	PRA 201	1.97 / 1.88
	PRA 202	5.95 / 3.37
	PRA 203	6.80 / 4.53
	PRA 204	9.29 / 9.57
	PRA 205	1.78 / 1.12
15	PRA 206	5.00 / 5.92
	PRA 207	1.58 / 2.15
	PRA 208	5.04 / 6.91

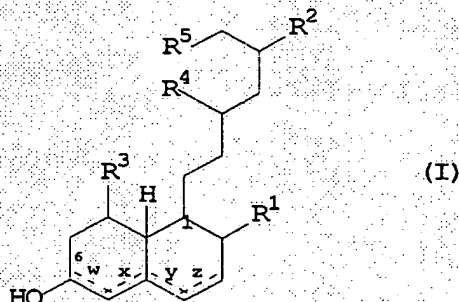
REFERENCES

1. M Arai et al, Sankyo Kenkyusho Nempo
(Annu. Rep. Sankyo Res. Lab.) 40: 1-38 (1988)
2. Hosobuchi et al, Biotechnology and Bioengineering,
5 42: 815-820 (1993).
3. EP-A-0,649,907.
4. US-A-3,983,140.
5. K. Wagschal et al, J. Chem. Soc., Perkin Trans.
1: 2357-2363 (1996)
- 10 6. EP-A-0,215,665
7. Okazaki et al, Annu.Rep.Sankyo Res. Lab. 41: 123-133
(1989)
8. Hopwood et al, Genetic Manipulation of Streptomyces,
a Laboratory Manual, John Innes Foundation, Norwich,
15 1985.
9. Watanabe et al, Gene 163: 81-85 (1995)
10. P.J. Punt and C.A.M. van den Hondel, Methods in
Enzymology 216: 447-457 (1993)
11. Nara et al, Curr. Genet. 23: 28-32 (1993)
- 20 12. Yelton et al, Proc.Natl.Acad.Sci. USA 81: 1470-1474
(1984)
13. Sambrook et al, Molecular Cloning, a Laboratory
Manual, Cold Spring Harbor Laboratory, Cold Spring
Harbor, New York (1989)
- 25 14. EP-A-0,635,574

CLAIMS

1. A process for preparing a compound of formula

(I)



5 wherein:

each of R^1 and R^2 independently represent a hydrogen atom, a hydroxy, C_{1-10} alkyl, C_{1-10} alkoxy, C_{3-10} cycloalkyl or C_{6-10} aryl group or a C_{7-11} aralkyl group optionally containing one or more hetero atoms;

10 R^3 represents R^1CO- or $R^1C(O)O-$;

each of R^4 and R^5 independently represent a hydrogen atom, $-COOR^1$ (except that then R^1 is not a hydroxy or alkoxy group), $-OR^1$ or $-COR^1$ or, when combined complete a six-membered carbon ring having one or two oxygen

15 heteroatoms;

wherein each of the alkyl, alkoxy, cycloalkyl, aryl and/or aralkyl groups can be optionally substituted with one or more halogen atoms, trifluoromethyl, hydroxy or C_{1-4} alkoxy groups;

20 and w , x , y and z represent none, 1 or when taken together up to 2 double bonds;

or a salt and/or isomer thereof,

the process comprising culturing a fungal host cell that expresses a foreign hydroxylase enzyme under conditions that allow biosynthesis to take place.

- 5 2. A process according to claim 1 wherein R^1 represents a methyl group, R^2 represents a hydroxy group and/or there are two double bonds at positions x and z.
3. A process according to claim 1 or 2 wherein R^3 represents a 2-methyl butyrate group, R^4 represents a hydroxy
10 group and R^5 represents -COOH.
4. A process according to any preceding claim wherein the compound is in HMG-CoA reductase inhibitor.
5. A process according to any preceding claim for preparing pravastatin (either in acid open ring or lactone
15 form) or a salt thereof.
6. A polynucleotide comprising a sequence encoding a hydroxylase enzyme operably linked to fungal expression signal(s) and/or fungal promoter.
7. A polynucleotide according to claim 6 wherein
20 the hydroxylase enzyme is of bacterial origin.
8. A polynucleotide according to claim 6 or 7 wherein the hydroxylase enzyme is from *Streptomyces*.
9. A polynucleotide according to any of claims 6 to 8 wherein the signal(s) and/or promoter are from
25 *Aspergillus* or *Penicillium*.
10. A polynucleotide according to any of claims 6 to 9 additionally comprising a pgk or gpd gene.
11. A vector comprising a polynucleotide as defined in any of claims 6 to 10.

12. A process for transfecting or transforming a host cell, the process comprising transforming or transfecting a fungal cell with a vector according to claim 11.

5 13. A process according to claim 12 wherein the host cell is co-transformed with another vector which contains a selectable or selective marker.

14. A process according to claim 13 wherein the marker confers resistance to either hygromycin or phleomycin.

10 15. A fungal host cell comprising a polynucleotide that encodes a foreign hydroxylase enzyme.

16. A host cell according to claim 15 obtainable by a process as defined in any of claims 12 to 14 or containing a polynucleotide as defined in any of claims 6 to 10.

15 17. A host cell according to claim 15 or 16 which is *Penicillium citrinum* or *Aspergillus terreus*.

18. A host cell according to any of claims 15 to 17 which is capable of biosynthesising, *de novo*, a compound of formula (I).

20 19. A host cell according to any of claims 15 to 18 which is capable of biosynthesising a compound of formula (II) (which is formula (I) where OH at the 6-position is replaced by H) and converting it, by the hydroxylase enzyme, into a compound of formula (I).

25 20. A host cell according to any of claims 15 to 19 which can be used as the fungal host cell in a process as defined in any of claims 1 to 5.

21. A compound of formula (I) produced either by a process according to any of claims 1 to 5 or by a fungal host cell according to any of claims 15 to 20.

22. A pharmaceutical composition comprising a
5 compound according to claim 21 and a pharmaceutically acceptable carrier or excipient.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 98/05362

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12P17/06 C12P7/62 C12N9/02 C12N15/80
C12N1/15 A61K31/215 A61K31/365 //(C12N1/15,C12R1:80,1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WATANABE, ICHIRO ET AL: "Cloning, characterization and expression of the gene encoding cytochrome P-450sca-2 from Streptomyces carbophilus involved in production of pravastatin, a specific HMG-CoA reductase inhibitor" GENE (1995), 163(1), 81-5 CODEN: GENED6; ISSN: 0378-1119, XP004041957	1
X	see the whole document	21,22
Y	EP 0 465 189 A (LILLY CO ELI) 8 January 1992	1,10,13, 14
X	see claims	6-9,11, 12, 15-17,22
	--- -/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

6 January 1999

Date of mailing of the international search report

18/01/1999

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Authorized officer

Delanghe, L

INTERNATIONAL SEARCH REPORT

Int'l Application No.
PCT/EP 98/05362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 388 166 A (OJI PAPER CO) 19 September 1990	6,7, 10-12, 15,16 13,14
Y	see claims	
X	WO 93 08287 A (MERCK & CO INC) 29 April 1993	6-9,11, 12,15-17
Y	see page 32 - page 33; claims	10,13,14
Y	CHEMICAL ABSTRACTS, vol. 118, no. 23, 7 June 1993 Columbus, Ohio, US; abstract no. 226810, NARA, FUTOSHI ET AL: "Development of a transformation system for the filamentous, ML-236B (compactin)-producing fungus Penicillium citrinum" XP002089171 see abstract & CURR. GENET. (1993), 23(1), 28-32 CODEN: CUGED5;ISSN: 0172-8083,1993,	10,13,14
Y	CHEMICAL ABSTRACTS, vol. 117, no. 23, 7 December 1992 Columbus, Ohio, US; abstract no. 227792, NARA, FUTOSHI ET AL: "Plasmids for transformation of filamentous fungi" XP002089172 see abstract & JP 03 262486 A (SANKYO CO., LTD., JAPAN)	10,13,14
Y	SMITH, ANDREW W. ET AL: "Analysis of promoter activity by transformation of Acremonium chrysogenum" GENE (1992), 114(2), 211-16 CODEN: GENED6;ISSN: 0378-1119,1992, XP002089170 see the whole document	10,13,14
A	WO 96 40863 A (MASSACHUSETTS INSTITUTE OF TECHNOLOGY, USA) 19 December 1996	1
X	see claims	21,22
A	EP 0 649 907 A (SQUIBB BRISTOL MYERS CO) 26 April 1995	1
X	see claims	21,22
A	EP 0 215 665 A (SANKYO CO) 25 March 1987	1
X	see claims	21,22
A	GB 2 077 264 A (SANKYO CO) 16 December 1981	1
X	see claims	21,22
-/--		

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/05362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE WPI Section Ch, Week 8534 Derwent Publications Ltd., London, GB; Class B03, AN 85-206666 XP002052752 & JP 60 130548 A (ENDO A), 12 July 1985</p>	1
X	<p>see abstract</p>	22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 98/05362

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application as follows :

1. Claims : 1-5,18,19,21,22 and 20 partially

PREPARATION OF A COMPOUND OF FORMULA I BY CULTURING A FUNGAL HOST CELL EXPRESSING A FOREIGN HYDROXYLASE ENZYME

2. Claims : 6-17 and 20 partially

A POLYNUCLEOTIDE ENCODING A HYDROXYLASE ENZYME OPERABLY LINKED TO A FUNGAL EXPRESSION SYSTEM

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/05362

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0465189 A	08-01-1992	CA 2045951 A JP 6007172 A	07-01-1992 18-01-1994
EP 0388166 A	19-09-1990	JP 3015392 A JP 2242685 A CA 2012025 A DE 69025081 D DE 69025081 T	23-01-1991 27-09-1990 14-09-1990 14-03-1996 30-05-1991
WO 9308287 A	29-04-1993	AU 657800 B AU 2701692 A BG 98714 A CA 2080573 A CN 1074484 A CZ 9400884 A EP 0540210 A EP 0856516 A FI 941730 A HU 69783 A JP 2655790 B JP 6113884 A MX 9205902 A NO 941345 A NZ 244714 A PL 172155 B SK 43194 A US 5559005 A US 5629171 A ZA 9207906 A	23-03-1995 22-04-1993 28-02-1995 16-04-1993 21-07-1993 15-03-1995 05-05-1993 05-08-1998 14-04-1994 28-09-1995 24-09-1997 26-04-1994 30-06-1994 15-06-1994 25-03-1994 29-08-1997 06-11-1996 24-09-1996 13-05-1997 03-06-1994
WO 9640863 A	19-12-1996	CA 2223122 A EP 0836642 A	19-12-1996 22-04-1998
EP 0649907 A	26-04-1995	AU 679113 B AU 7597294 A CA 2134025 A CN 1106067 A FI 944926 A HU 69959 A IL 111084 A JP 7184670 A SG 47504 A	19-06-1997 11-05-1995 23-04-1995 02-08-1995 23-04-1995 28-09-1995 04-01-1998 25-07-1995 17-04-1998
EP 0215665 A	25-03-1987	DE 3682557 A JP 1696791 C JP 3066297 B JP 62174040 A US 5153124 A US 5272174 A JP 1790339 C JP 4082135 B JP 62190094 A	02-01-1992 28-09-1992 16-10-1991 30-07-1987 06-10-1992 21-12-1993 29-09-1993 25-12-1992 20-08-1987
GB 2077264 A	16-12-1981	JP 1347361 C JP 57002240 A JP 61013699 B JP 1500955 C JP 57108039 A	13-11-1986 07-01-1982 15-04-1986 28-06-1989 05-07-1982

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/EP 98/05362

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2077264 A		JP 63048858 B	30-09-1988
		JP 1454452 C	25-08-1988
		JP 57050894 A	25-03-1982
		JP 62054476 B	16-11-1987
		JP 1475008 C	18-01-1989
		JP 57067575 A	24-04-1982
		JP 63021672 B	09-05-1988
		AT 374495 B	25-04-1984
		AT 256781 A	15-09-1983
		AU 549988 B	27-02-1986
		AU 7137681 A	10-12-1981
		BE 889150 A	09-12-1981
		CA 1150170 A	19-07-1983
		CH 655090 A	27-03-1986
		DE 3122499 A	24-12-1981
		DK 247081 A,B,	07-12-1981
		FI 811762 A,B,	07-12-1981
		FR 2483912 A	11-12-1981
		IE 51270 B	26-11-1986
		MX 9203563 A	01-09-1992
		NL 8102737 A,B,	04-01-1982
		SE 453389 B	01-02-1988
		SE 8103560 A	07-12-1981
		US 4346227 A	24-08-1982
		US 4410629 A	18-10-1983
		US 4448979 A	15-05-1984